Retrospective Diagnosis of Fatal BP180-Deficient Non-Herlitz Junctional Epidermolysis Bullosa Suggested by Immunofluorescence (IF) Antigen-Mapping of Parental Carriers Bearing Enamel Defects

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TO THE EDITOR

The genetic blistering disorder epidermolysis bullosa (EB) is divided into three major types, EB simplex, junctional EB (JEB) and dystrophic EB (Fine *et al.*, 2000) caused by mutations in at least 10 genes (Uitto and Richard 2004). JEB is characterized by blistering in the lamina lucida resulting from mutations in one of the following six genes: *LAMA3*, *LAMB3*, and *LAMC2* that encode the subunit polypeptides of laminin-332 or *ITGA6*, *ITGB4*, or *COL17A1* that encode transmembrane proteins of the hemidesmosome, integrin $\alpha 6\beta 4$ and BP180 (type XVII collagen), respectively (Varki *et al.*, 2006). Partial deficiency of laminin-332 or integrin $\alpha 6\beta 4$, or complete deficiency of BP180 cause generalized non-Herlitz JEB (nH-JEB, MIM 226650) (Jonkman *et al.*, 1995; McGrath *et al.*, 1995), clinically identified by skin blistering after minor trauma, non-scarring alopecia, nail hypoplasia, enamel pitting (amelogenesis imperfecta), and, in some patients, EB nevi (Hintner and Wolff 1982). Immunofluorescence antigen mapping (IFM) of the patients' skin is a helpful technique in determining the level of blister formation and identifying the deficient protein.

Here, we report the use of IFM to identify the subtype of EB using skin from the parents of two deceased probands. The parents lived in Iraq and were first cousins (Figure 1a, III-6 and III-7). Their first child (IV-3), a son, born in 1992 in Iraq, had no skin problems. Subsequently, the family had to move to Iran. Their second son (IV-4), born in 1995 in Tehran, had a widespread blistering skin condition thought to be EB (Figure 1e). He spent all his life in hospital receiving bandages and antibiotics, dying aged 9 months. He had no history of hoarseness. The next child (IV-5), a daughter, also had congenital blistering and died in hospital at the age of 1 month. In 2000, the family moved as refugees to Australia. They were referred for possible prenatal diagnosis in 2002.

The parents denied any previous family history of EB and they themselves had no skin problems. On examination, their skin and hair were normal; however, they had dental anomalies, first noted in the father at the age of 9 years (Figure 1b). The father's teeth showed significant pitting and horizontal ridging with extensive loss of enamel. The mother's teeth had horizontal ridging (Figure 1c and Figure S1). Their 13-year-old son's teeth showed extensive enamel mottling (Figure 1d). Roughly, half the parents' siblings, five of a total of nine, had abnormal teeth by history. The studies were conducted with institutional approval of the experiments, patient consent, and adherence to the Declaration of Helsinki Principles.

Skin biopsies were taken from the abdomen of the father and mother in 2002 and tested by IFM with a panel of monoclonal antibodies (Yiasemides *et al.*, 2006). IFM showed no reduction in staining with antibodies to either type VII collagen (LH7.2), laminin-332 (D4B5; K140; BM165), integrin $\alpha 6\beta 4$ (GOH3; 439-9B), or BP180 (233) and LAD-1 (123). On the basis of the abnormal dentition, it was felt that the deceased children had EB, most probably the junctional variant. Therefore, *LAMA3*, *LAMB3*, and *LAMC2* were screened but yielded negative results. Subsequently, the antibodies 1D1 to the distal C-terminal ectodomain of BP180 (epitope residues 1357–1387) and 1A8c to the N-terminal endodomain (epitope residues 155–163) (Owaribe *et al.*, 1991) (kind gift of Dr Katsushi Owaribe, Nagoya, Japan) were tested against the parents' skin and the immunofluorescence signal was found to be significantly reduced compared with normal human skin in both the mother and father (Figures 2a–d). Thereafter, screening of *COL17A1* with forward primer FEX10: 5′-GCTTTCAATCCCAAACCAGG-3′ and reverse primer REX10: 5′-TGGCAAACATTCTGAGGGTC-3′ revealed the heterozygous presence of a novel deletion mutation *COL17A1* c.823delA in exon 10 – numbering according to Giudice *et al.* (1992) – in DNA from both parents and the older, clinically unaffected son (Figures 2e and f). Elaborate DNA screening of the parents showed the absence of glycine substitutions. Both parents were heterozygous for the single nucleotide polymorphisms c.734C/T (p.T/M) and c.1168T/C (p.A/A). In addition, the mother was heterozygous for the single nucleotide polymorphisms c.2212G/A (p.V/M) and c.3009G/A (p.P/P). The deletion of A at position c.823 was predicted to result in a frame-shift and a novel mis-sequence beginning at amino acid p.239 and to conclude in a PTC at position p.291 (*COL17A1*:c.823delA, p.T239fs52). Exon 10 codes for part of the intracellular domain of BP180. Thus, most likely the deceased children had a severe form of non-Herlitz JEB due to the homozygous presence of the c.823delA *COL17A1* mutation.

The mother became pregnant again in mid-2003 and a prenatal chorionic

Abbreviations: EB, epidermolysis bullosa; IFM, immunofluorescence antigen mapping; JEB, junctional EB; nH-JEB, non-Herlitz junctional epidermolysis bullosa

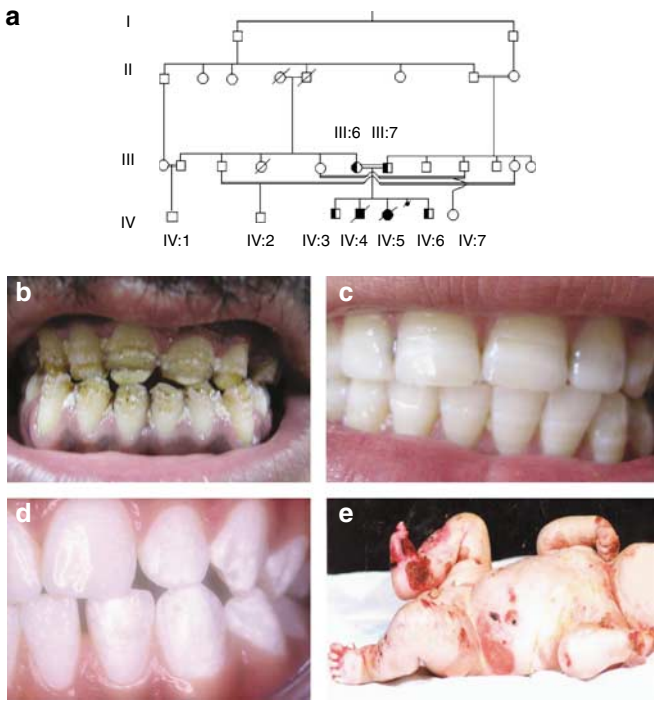

Figure 1. Clinical features of the family. (**a**) The pedigree indicates the consanguinity of the family members. (**b**) The heterozygous father's (III-7) teeth had extensive pitting and horizontal ridging of the incisors and loss of enamel in the molars; (**c**) the heterozygous mother's (III-6) teeth had horizontal ridging of the enamel; (**d**) their oldest son's (IV-3) teeth showed white mottling of the enamel. (**e**) The baby born in 1995 (IV-4) had generalized blistering.

villous sampling was performed at 10 weeks' gestation, which revealed a male infant (IV-6) who was a heterozygous carrier of the mutation and was phenotypically normal at birth. Repeat skin biopsies were performed on the parents and also of their older son (IV-3) to analyze the consequences of the mutation at mRNA and protein levels. Keratinocytes were grown from the skin biopsies and mRNA was extracted (Jonkman *et al.*, 1997; Molnar *et al.*, 2000). To investigate the possible presence of aberrant mRNA transcripts around the c.823delA mutation, RT-PCR was performed using the following oligonucleotides: forward primer F0529: 5′- ATTCGAGTTCGACTGCAG AG-3′ (exon 8) and reverse primer R1204 5′-TGTCCTTGGTCATGAT GAGC-3′ (exon 14). No alternatively spliced mRNA transcripts were detected on agarose gel (Figure S2). Subsequently, the amplified products of 676 bp were purified and sequenced. The sequence analysis for the heterozygous parents and their older son only showed mRNA transcribed from the

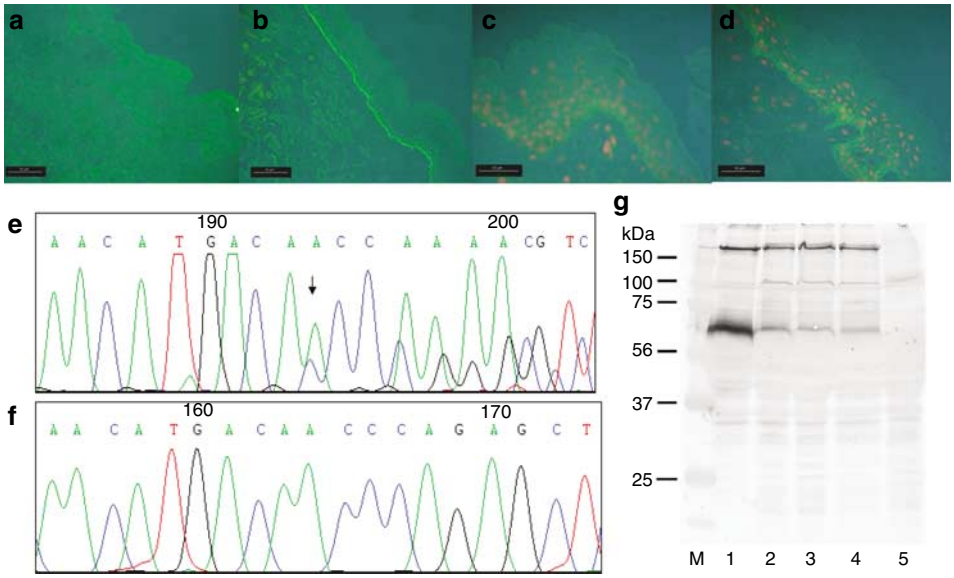

Figure 2. Immunofluorescence staining (bar = 50 μm) of BP180 with monoclonal antibody 1D1 of the skin of the parents of the deceased probands (**a**: mother) was reduced compared with (**b**) normal human control skin. Immunofluorescence staining (original magnification × 200) of BP180 with monoclonal antibody 1A8c of the skin of the parents of the deceased probands (**c**: father) was also reduced compared with (**d**) normal human control skin. Mutation analysis revealed the heterozygous presence of the c.823delA mutation in the *COL17A1* gene in the DNA of the parents and of the two sons alive (**e**: father). (**e**) Owing to the deletion of an A (arrow) in exon 10 a frameshift occurs finally resulting in a PTC. (**f**) In DNA of normal human control skin, the 1-bp deletion is absent. (**g**) Immunoblot of cultured keratinocytes using monoclonal antibody 1A8c against the intracellular domain of BP180 reveals reduced expression of normal length BP180 protein and the 60 kDa cytoplasmic domain of BP180 after the shedding of the ectodomain in the parental and sib carriers. Lanes: (M) molecular weight marker, (1) healthy control, (2) son (IV-3), (3) father, (4) mother, and (5) BP180-negative nH-JEB control.

wild-type allele, as the mRNA from the mutated strand was most likely degraded by nonsense-mediated RNA decay (Figure S3). Similarly, immunoblotting of keratinocyte extracts (van Leusden *et al.*, 2001) using 1A8c antibody revealed reduced expression of full-length BP180 in the carriers (Figure S4), and of the 60-kDa cytoplasmic domain of BP180 after the ectodomain has been shed (Figure 2g (Hirako *et al.*, 1998)). No smaller BP180 molecule of 290 amino acids was detected, excluding the possibility of a shortened BP180 molecule that could exert a dominant-negative effect on the wild-type BP180.

In summary, the testing showed (i) reduced expression of BP180 by monoclonal antibodies 1D1 and 1A8c but not by 233 or 123 in heterozygous carriers of the mutation; (ii) a new mutation, *COL17A1*:c.823delA,p.T239fs52 (Figure S5), which is postulated to result in nonsense-mediated mRNA decay, as there was no stable expression of a mRNA transcript bearing the mutation or of a smaller BP180 protein; and (iii) no aberrant splicing of exon 10 due to the mutation as the only produced BP180 was of normal size and no smaller mRNA transcripts were detected.

A normal male infant (IV-6) was born in Sydney in March 2005, who was a heterozygous carrier at the DNA level. By April 2006 four primary teeth had erupted, and thus far (August 2006) no enamel defects have manifested.

There are several unusual features of this family. First, the two babies with nH-JEB due to a *COL17A1* mutation had died despite being hospitalized. Rarely, patients with this type of nH-JEB die in early life (Hintner and Wolff 1982; Abu Sa'd *et al.*, 2006; Varki *et al.*, 2006). It is possible that in a more advanced healthcare setting, the children might have survived. The majority of BP180-deficient nH-JEB cases owing to PTC mutations in *COL17A1* do survive to adulthood, although the phenotype can vary significantly amongst these, with some having anemia and growth retardation (Varki *et al.*, 2006).

Secondly, although the reduction of BP180 in carriers of *COL17A1* mutations was reported by us before (Pasmooij *et al.*, 2005, 2006, 2007). It is remarkable that IMF has been used to suggest the diagnosis of this form of nH-JEB in carriers where the probands were already deceased. It is not very uncommon in Australia for families to be referred for diagnosis only after an infant dies from EB, before adequate investigations have been performed on the infant (DF Murrell, personal experience) and hence IFM of the parents can be useful to direct appropriate investigations. Carriers of recessive PTC mutations of *COL7A1* in recessive dystrophic EB cannot be detected using LH7:2 or other antibodies against collagen VII (Bruckner-Tuderman *et al.*, 1988). Furthermore, electron microscopy shows a reduced number of anchoring fibrils in carriers of dystrophic EB and of hemidesmosomes in JEB, but they are not reliable enough for diagnosis (Tidman and Eady 1986). One limitation of the use of IFM is that the only antibodies which gave reduced staining, 1D1 and 1A8c, are not commercially available and are in limited supply (Owaribe, personal communication, 2006); however, the monoclonal V58 to residues 234–398 of the intracellular domain recently became commercially available (Olaru *et al.*, 2006).

Thirdly, the presence of enamel defects in carriers of a deletion mutation in *COL17A1* is of interest. The previously reported mutations causing dental abnormalities in heterozygous carriers of *COL17A1* mutations were all dominant glycine-substitution mutations, G609D (Tasanen *et al.*, 2000a), G612R (Tasanen *et al.*, 2000a), G627V (McGrath *et al.*, 1996), and G633D (Tasanen *et al.*, 2000b), yet there were no glycine substitutions in these parents. In a report on mutations in *COL17A1* and *LAMB3* causing JEB in a family, there were two half-sisters of a proband who were heterozygous for the *COL17A1* mutation C.3781C>T; p.R1226X, who were reported to have dental abnormalities (Floeth and Bruckner-Tuderman 1999). Enamel defects with these appearances (amelogenesis imperfecta) could also be caused by one of several enamel gene defects in this consanguineous family, unrelated to EB (Masuya *et al.*, 2005; Nakamura *et al.*, 2006). However, enamel defects could also be because of an environmental effect causing chronological hypoplasia of enamel during the period 0–8 years, when enamel is forming. This may include an acute childhood illness with prolonged high temperatures or chronic illness, such as malnutrition or exposure to high doses of fluoride, such as from rural well water. If the amelogenesis imperfecta were because of the heterozygous *COL17A1* mutation, haploinsufficiency would be implicated as the mutant mRNA most probably is degraded. Although there were differences between the parents' *COL17A1* polymorphisms, there is no evidence that these have caused enamel defects. Enamel formation is very sensitive and may not have redundancies that exist in skin to cope with the loss of one allele. However, in a recent study we found no enamel abnormalities in carriers of *COL17A1*-null mutations (Pasmooij *et al.*, 2007). Nevertheless, nH-JEB patients with reduced expression of BP180, who have mild localized blistering and normal scalp hair, all had at least enamel pitting (Pasmooij *et al.*, 2007). In some patients with nH-JEB, the dental anomalies may not be visible in probands until the primary molars have erupted at the age of 2–3 years (Nakamura *et al.*, 2006) (MF Jonkman, unpublished data). Clearly, the presence of dental abnormalities in these patients is a complex process and may involve a gene–environment interaction during development.

In conclusion, these unique cases of nH-JEB provide several useful insights into genotype–phenotype correlation: (a) nH-JEB caused by *COL17A1* mutations may be lethal in the first year of life; (b) diagnosis of BP180-deficient nH-JEB can be made from IFM of heterozygous carrier parents with 1D1 or 1A8c antibodies, at least for the c.823delA mutation; and (c) the novel mutation *COL17A1*:c.823delA results in a downstream PTC with nonsense-mediated decay of the abnormal transcript. The finding of enamel defects in the heterozygous carriers was difficult to explain by purely the reduction in BP180 protein expression, and may have been owing to other factors.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Web resources

Accession numbers, and URLs for data presented herein are as follows: GenBank, http://www.ncbi.nlm.nih.gov/GenBank/ (for human COL17A1 mRNA sequence (accession number NM_000494), human COL17A1 transcript sequence (accession number NP_000485) and sequences of exons 1–56 of COL17A1 (accession numbers U76564–U76604)).

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SUPPLEMENTARY MATERIAL

Figure S1. This picture illustrates the horizontal ridging of the enamel in the incisors of the mother (III:6).

Figure S2. RT-PCR of exon 10:The gel shows bands generated by RT-PCR of exon 10 of *COL17A1* from mRNA extracted from skin biopsies.

Figure S3. RT-PCR performed on mRNA of cultured keratinocytes of the heterozygous father and mother showed the absence of mRNA from the c.823delA mutation-bearing allele.

Figure S4. Immunoblotting of similar amounts of cultured keratinocyte extracts from the brother (1), father (2), mother (3), and normal control (4) showing a normal size 180 kd band of the BP180 protein in each.

Figure S5. Schematic diagram of the exons (gray and black) in COL17A1 showing the location of all previously reported mutations, with the red arrow indicating the mutation in this report in exon 10.

REFERENCES

Abu Sa'd J, Indelman M, Pfendner E, Falik-Zaccai TC, Mizrachi-Koren M, Shalev S *et al.* (2006) Molecular epidemiology of hereditary epidermolysis bullosa in a Middle Eastern population. *J Invest Dermatol* 126:777–81

Bruckner-Tuderman L, Ruegger S, Odermatt B, Mitsuhashi Y, Schnyder UW (1988) Lack of type VII collagen in unaffected skin of patients with severe recessive dystrophic epidermolysis bullosa. *Dermatologica* 176:57–64

Fine J-D, AJ ER, Bauer EA, Briggaman RA, Bruckner-Tuderman L, Christiano A *et al.* (2000) Revised classification system for inherited epidermolysis bullosa: report of the Second International Consensus Meeting on diagnosis and classification of epidermolysis bullosa. *J Am Acad Dermatol* 42:1051–66

Floeth M, Bruckner-Tuderman L (1999) Digenic junctional epidermolysis bullosa: mutations in *COL17A1* and *LAMB3* genes. *Am J Hum Genet* 65:1530–7

Giudice GJ, Emery DJ, Diaz LA (1992) Cloning and primary structural analysis of the bullous pemphigoid autoantigen BP180. *J Invest Dermatol* 99:243–50

Hintner H, Wolff K (1982) Generalized atrophic benign epidermolysis bullosa. *Arch Dermatol* 118:375–84

Hirako Y, Usukura J, Uematsu J, Hashimoto T, Kitajima Y, Owaribe K (1998) Cleavage of BP180, a 180-kDa bullous pemphigoid antigen, yields a 120-kDa collagenous extracellular polypeptide. *J Biol Chem* 273:9711–7

Jonkman MF, Scheffer H, Stulp R, Pas HH, Nijenhuis M, Heeres K *et al.* (1997) Revertant mosaicism in epidermolysis bullosa caused by mitotic gene conversion. *Cell* 88:543–51

Jonkman MF, de Jong MC, Heeres K, Pas HH, van der Meer JB, Owaribe K *et al.* (1995) 180 kD bullous pemphigoid antigen (BP180) is deficient in generalized atrophic benign epidermolysis bullosa. *J Clin Invest* 95:1345–52

Masuya H, Shimizu K, Sezutsu H, Sakuraba Y, Nagano J, Shimizu A *et al.* (2005) Enamelin (Enam) is essential for amelogenesis: ENU-induced mouse mutants as models for different clinical subtypes of human amelogenesis imperfecta (AI). *Hum Mol Genet* 14:575–83

McGrath JA, Gatalica B, Christiano AM, Li K, Owaribe K, McMillan JR *et al.* (1995) Mutations in the 180-kD bullous pemphigoid antigen (BPAG2), a hemidesmosomal transmembrane collagen (COL17A1), in generalized atrophic benign epidermolysis bullosa. *Nat Genet* 11:83–6

McGrath JA, Gatalica B, Li K, Giles M, Dunnill S, McMillan JR *et al.* (1996) Compound heterozygosity for a dominant glycine substitution and recessive internal duplication mutation in the type XVII collagen gene results in junctional epidermolysis bullosa and abnormal dentition. *Am J Pathol* 148: 1787–96

Molnar K, van der Steege G, Jonkman MF, Nijenhuis M, Husz S, van der Meer JB *et al.* (2000) Two type XVII collagen (BP180) mRNA transcripts in human keratinocytes: a long and a short form. *Clin Exp Dermatol* 25:71–6

Nakamura H, Sawamura D, Goto M, Nakamura H, Kida M, Ariga T *et al.* (2006) Analysis of the COL17A1 in non-Herlitz junctional epidermolysis bullosa and amelogenesis imperfecta. *Int J Mol Med* 18:333–7

Olaru F, Mihai S, Petrescu I, Zillikens D, Sitaru C (2006) Generation and characterization of monoclonal antibodies against the intracellular domain of hemidesmosomal type XVII collagen. *Hybridoma (Larchmt)* 25: 158–62

Owaribe K, Nishizawa Y, Franke WW (1991) Isolation and characterization of hemidesmosomes from bovine corneal epithelial cells. *Exp Cell Res* 192:622–30

Pasmooij AMG, Jonkman MF, Pas HH, Klingberg S, Pfendner E, Uitto J *et al.* (2005) Dental abnormalities in heterozygous carriers of the *COL17A1* 823 del A mutation in a family with non-Herlitz junctional EB. *JEADV* (Suppl 2), FC03.9, p 12, November 2005

Pasmooij AMG (2006) *Genotyping of Unusual Phenotypes in Epidermolysis Bullosa*. Department of Dermatology, University Medical Center Groningen, The Netherlands: University of Groningen, 201

Pasmooij AMG, Pas HH, Jansen GHL, Lemmink HH, Jonkman MF (2007) Localized and generalized forms of blistering in junctional epidermolysis bullosa due to COL17A1 mutations in The Netherlands. *Br J Dermatol* in press

Tasanen K, Floeth M, Schumann H, Bruckner-Tuderman L (2000a) Hemizygosity for a glycine SUBSTITUTION in collagen XVII: unfolding and degradation of the ectodomain. *J Invest Dermatol* 115:207–12

Tasanen K, Floeth M, Schumann H, Bruckner-Tuderman L (2000b) Hemizygosity for a glycine substitution in collagen XVII: unfolding and degradation of the ectodomain. *J Invest Dermatol* 115:207–12

Tidman MJ, Eady RA (1986) Structural and functional properties of the dermoepidermal junction in obligate heterozygotes for recessive forms of epidermolysis bullosa. *Arch Dermatol* 122:278–81

Uitto J, Richard G (2004) Progress in epidermolysis bullosa: genetic classification and clinical implications. *Am J Med Genet C Semin Med Genet* 131C:61–74

van Leusden MR, Pas HH, Gedde-Dahl TJ, Sonnenberg A, Jonkman MF (2001) Truncated type XVII collagen expression in a patient with non-herlitz junctional epidermolysis bullosa caused by a homozygous splice-site mutation. *Lab Invest* 81:887–94

Varki R, Sadowski S, Pfendner E, Uitto J (2006) Epidermolysis bullosa. I. Molecular genetics of the junctional and hemidesmosomal variants. *J Med Genet* 43:641–52

Yiasemides E, Walton J, Villanueva E, Marr P, Murrell DF (2006) A comparative study between electron microscopy and immunofluorescence mapping in the diagnosis of epidermolysis bullosa. *Am J Dermatopath* 28:i:387–94